

# Integrative taxonomy revealed a new species of *Lefua* (Teleostei, Nemacheilidae) from Fukui Prefecture, Japan

Yuta Katayama<sup>1</sup>, Naoto Sawada<sup>1,2</sup>

<sup>1</sup> Department of Zoology, Graduate School of Science, Kyoto University, Kitashirakawa-oiwakecho, Sakyo-ku, Kyoto-shi, Kyoto, 606-8502 Japan

<sup>2</sup> Department of Biological Sciences, School of Science, The University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

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Corresponding author: Naoto Sawada (sawada@zoo.zool.kyoto-u.ac.jp)

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## Abstract

Eight-barbel loaches belonging to the genus *Lefua* have diverged into seven species in freshwaters of East Asia. Recent studies have discovered a new population in the Kuzuryu River drainage system of Fukui Prefecture, central Japan. Based on the results of the genetic analyses and morphological comparisons, we describe this population as a new species, *Lefua nishimurai* **sp. nov.** Body width, interorbital width, orbit diameter, preanal length, snout length, and the newly examined head width greatly contributed to the discrimination between *L. torrentis*, *L. tokaiensis* and *L. nishimurai* **sp. nov.** The new species can be distinguished from other congeners by combining the following characteristics: 1) eyes positioned dorsally on the head; 2) a narrow conspicuous longitudinal mark between the base of the outer rostral barbel and the eye; 3) small dark spots on the body, dorsal, and caudal fins; 4) a small dorsal fin and eye diameter; and 5) black spots above and below the base of the caudal fin. Based on the phylogenetic relationships of the genus, *L. nishimurai* **sp. nov.** is estimated to have diverged early from its common ancestor in central Japan. Understanding the natural history of this new species and implementing conservation measures are crucial because of its narrow, fragmented distribution and presence in vulnerable habitats.

## Key Words

endangered species, freshwater fish, inland water, nemacheilid loach, nuclear phylogeny, species richness

## Introduction

*Lefua* Herzenstein, 1888, is a genus of eight-barbel loach belonging to the family Nemacheilidae, and its members are widely distributed in Mongolia, Russia, China, Korea, and Japan (Hosoya 2013; Kottelat 2013; Ito et al. 2019). The genus has been characterized by rounded caudal fins, elongated anterior nostrils forming long nasal barbels, and the absence of a postcleithrum and epural (Nichols 1943; Sawada 1982; Prokofiev 2005). The genus *Lefua* was originally established as a replacement name for the genus *Octonema* Herzenstein, 1887. *Octonema* was erected for *Diplophysa costata* Kessler, 1876, and *O. pleskei* Herzenstein, 1887, which are indigenous to Korea, China, and Russia, while its name was preoccupied by the piscine and coelenterate genera (Kottelat 2012). Subsequently,

in the early 1900s, three species, *L. nikkonis* (Jordan & Fowler, 1903), *L. echigonia* Jordan & Richardson, 1907, and *L. sayu* (Herre & Lin, 1936), were described in China and Japan. After recent descriptions of two new species, *L. torrentis* Hosoya, Ito & Miyazaki, 2018 and *L. tokaiensis* Ito, Hosoya & Miyazaki, 2019, from Japan, seven species have been accepted in the genus (Ito et al. 2019).

*Lefua* species were mainly distinguished in the early 1900s based on a combination of the following characteristics: coloration of the caudal fin base, dorsal fin, and mature male body, as well as vertebral count and head size (Naseka and Bogutskaya 2004; Ito et al. 2019; Ohgita and Matsunuma 2020). Subsequently, Hosoya (1993) examined snout coloration, body elongation, and eye size in *L. echigonia*, and discovered an unidentified species (currently *L. torrentis*). Molecular phylogenetic relation-

ships among Japanese *Lefua* species were clarified in several genetic studies in the 2000s (Sakai et al. 2003; Miha-  
ra et al. 2005; Miyazaki et al. 2007, 2011, 2018). These  
investigations also highlighted the genetic distinctiveness  
of *L. torrentis* and another genetic clade, presently iden-  
tified as *L. tokaiensis*. These findings substantiated the  
monophyly of a clade comprising the Japanese *L. echi-  
gonia*, *L. torrentis*, and *L. tokaiensis* with both mitochon-  
drial and nuclear loci. These two unidentified species  
were recently described based on detailed morphological  
comparisons that emphasized the importance of snout  
coloration and eye position (Hosoya et al. 2018; Ito et al.  
2019). According to integrative analyses conducted in the  
2000s, the species richness of Japanese *Lefua* and genetic  
relationships within the genus have been well elucidated.

The distribution of the Japanese *Lefua* has been per-  
sistently investigated in sequential studies. Recently,  
however, a new population of this genus was discovered  
in the Kuzuryu River drainage system of Fukui Prefec-  
ture, central Japan (Natural Environment Division, De-  
partment of Safety and Environment, Fukui Prefecture  
2016). Subsequent studies employing assessments of  
morphological features and mitochondrial phylogeny  
have indicated the morphological and genetic distinc-  
tiveness of the population, identifying them as *L. torren-  
tis* (Nishimura 2020; Nakajima et al. 2021). Katayama  
(2021) comprehensively surveyed the distribution of the  
genus in Fukui Prefecture and estimated a considerably  
fragmented habitat and a small population size of *Lefua*  
loaches with morphological features similar to those re-  
ported by Nakajima et al. (2021).

Based on the distinctive morphological and mitochon-  
drial phylogenetic traits, the *Lefua* population present in  
the Kuzuryu River system may be an independent spe-  
cies with unique evolutionary origins. Considering the  
mitochondrial introgression observed in the congeners  
(Miyazaki et al. 2018), however, it is imperative to elu-  
cidate the nuclear phylogenetic position of the popula-  
tion to accurately determine its taxonomic account. In the  
present study, we clarified the nuclear phylogenetic and  
morphological relationships between the population and  
congeners, and describe it as a new species.

## Materials and methods

### Taxon sampling and specimen preparation

A total of 92 *Lefua* specimens were newly collected from  
Japan (Table 1). Among them, specimens of *L. torrentis*  
and *L. tokaiensis* were obtained from a wide area, which  
covers the distribution of the two species and the genetic  
populations of *L. torrentis* (Fig. 1; Yamada 2015; Nakaji-  
ma and Uchiyama 2017; Miyazaki et al. 2018). Moreover,  
25 specimens of the new species were collected from the  
Kuzuryu River system. Following Soto and Burhanuddin  
(1995), each individual was euthanized using clove oil  
(NOW Foods, USA). Digital images were captured using

a SLT-A55V camera with a SAL1855 lens (Sony Corpo-  
ration, Japan), and the right pelvic fins of the specimens  
were cut and preserved in 99.5% ethanol. Subsequently,  
whole bodies were fixed in 10% neutral formalin solution.  
The preservation solution was replaced with an 80% eth-  
anol solution after formalin fixation. For morphological  
comparison, measurements and counts of the holotypes  
of *L. torrentis* preserved in the Department of Fisheries,  
Kindai University (KUN-P 45408) and *L. tokaiensis* at the  
National Museum of Natural History (NSMT-P 132821)  
were obtained from their original descriptions (Hosoya  
et al. 2018; Ito et al. 2019). Newly collected specimens  
were deposited at the Department of Fisheries, Faculty of  
Agriculture, Kyoto University (FAKU) and Lake Biwa  
Museum (LBM).

### Genetic analysis

Genomic DNA was extracted from seven specimens of  
*L. torrentis*, five specimens of *L. tokaiensis*, and nine  
specimens of the new species. DNA was extracted from  
the right pectoral fin following the procedure outlined by  
Okamoto et al. (2006) using a protein precipitation solu-  
tion (Promega Corporation, USA). The primers RPEX1F  
(5'-TGGCCTCTTCCTTGCCGTC-3') and RPEX2R  
(5'-AACTCGTCTGGCTTTTCGCC-3') (Miyazaki et  
al. 2011) were used. Step-down polymerase chain reac-  
tion and nucleotide sequencing of approximately 750 bp  
non-coding sections of the nucleic ribosomal S7 sub-  
unit gene were conducted according to the methods de-  
scribed by Miyazaki et al. (2018) using Ex Premier DNA  
Polymerase (Takara Bio Inc., Japan). The products were  
sequenced using an ABI 3130xl Genetic Analyzer (Ap-  
plied Biosystems, USA), and finally, 463 bp alignments  
were obtained. The sequences were deposited in the In-  
ternational Nucleotide Sequence Database Collabora-  
tion through the DNA Databank of Japan (LC790700–  
LC790720).

The phylogenetic relationships between the 21 new-  
ly obtained sequences and the 25 sequences of the five  
*Lefua* species provided by Miyazaki et al. (2011, 2018)  
were estimated (Table 1). Phylogenetic trees were recon-  
structed based on 452 bp alignments shared by all se-  
quences using maximum-likelihood (ML) with IQ-TREE  
v1.6.12 (Nguyen et al. 2015) and Bayesian inference  
(BI) with the MPI version of MrBayes v3.2.7 (Altekar  
et al. 2004; Ronquist et al. 2012). The best-fit models in  
the ML phylogenies were calculated as the HKY mod-  
el based on the Bayesian information criterion and the  
greedy algorithm using ModelFinder implemented in  
IQ-TREE (Kalyanamoorthy et al. 2017). The branch  
support of the ML phylogenetic tree was assessed using  
1,000 non-parametric bootstrap (BS) replicates. The best-  
fit models in the BI were selected as the HKY+G model  
under the Bayesian Information Criterion using Partition-  
Finder v2.1.1 (Lanfear et al. 2016). The tree was sampled  
every 100 generations from the four Markov chains in

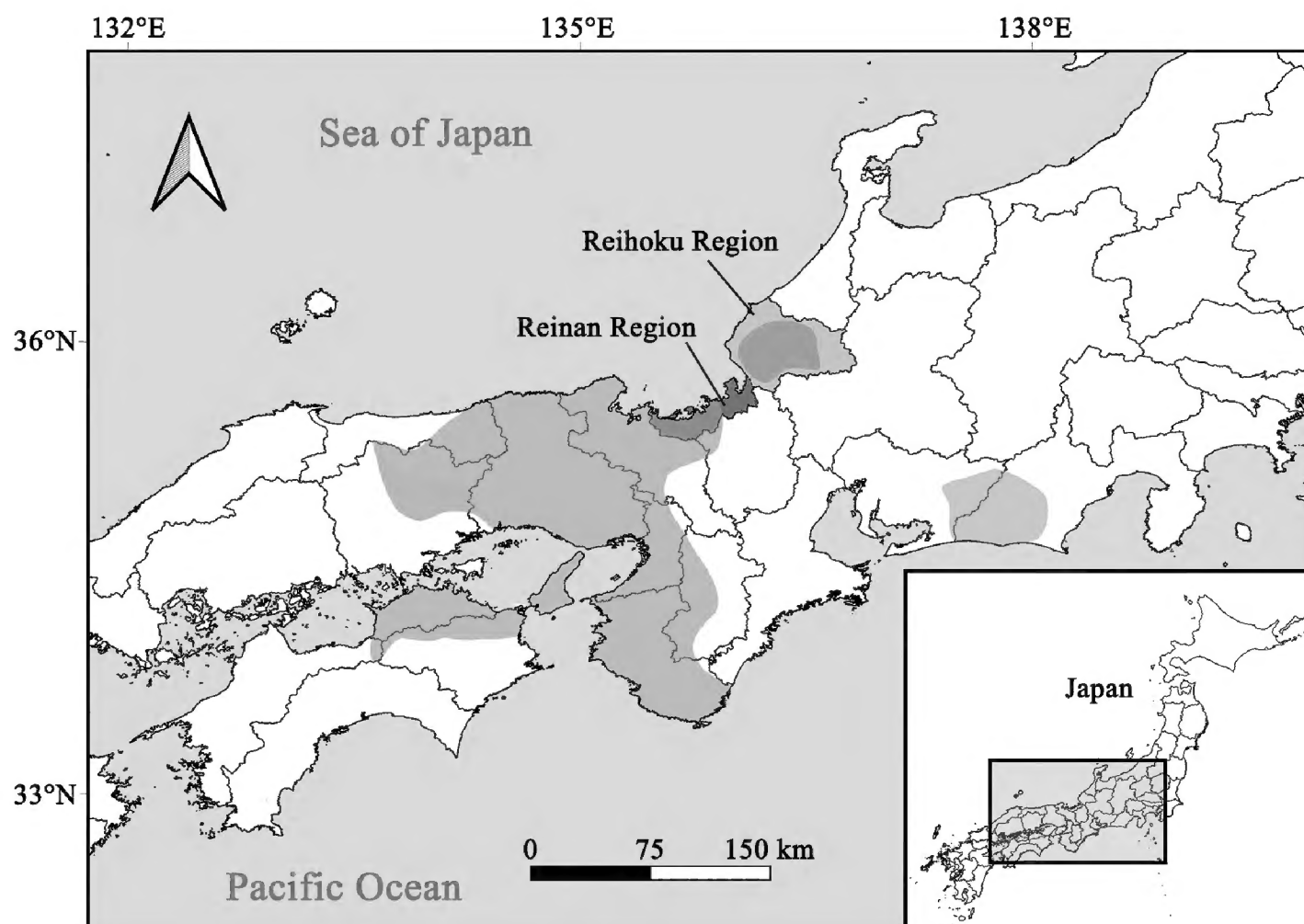
**Table 1.** Specimen list examined in this study with voucher numbers, collection localities, accession numbers of International Nucleotide Sequence Database (INSD), and references.

Voucher # (type status / population)	Collection locality	INSD Accession #	Reference
<i>L. torrentis</i>			
KUN-P45408 (holotype)	Kasuga, Hyogo		Hosoya et al. (2018)
FAKU 211461–211464 (Kii-Shikoku)	Aridagawa, Wakayama		This study
FAKU 211465–211468 (Kii-Shikoku)	Hidaka, Wakayama		This study
FAKU 211469–211477 (Kii-Shikoku)	Kitanada, Tokushima	LC790709	This study
FAKU 211478–211480 (Kii-Shikoku)	Gomyo, Kagawa	LC790710	This study
FAKU 211481–211484 (Sanyo)	Ayuya, Hyogo	LC790711	This study
FAKU 211485–211490 (Sanyo)	Jindaiurakabe, Hyogo		This study
FAKU 211491 (Sanyo)	Dojyo, Hyogo	LC790712	This study
FAKU 211492 (Sanyo)	Nagi, Okayama,	LC790713	This study
FAKU 211493–211499 (Sanyo)	Santo, Hyogo		This study
FAKU 211500 (Unidentified)	Yamada, Fukui	LC790714	This study
FAKU 211501–211506 (Nihonkai)	Taneji, Kyoto	LC790715	This study
(Sanyo)	Aoi, Kyoto	AB586659	Miyazaki et al. (2011)
(Sanyo)	Nose, Osaka	AB586660	Miyazaki et al. (2011)
(Kii-Shikoku)	Hidaka, Wakayama	AB586662	Miyazaki et al. (2011)
(Kii-Shikoku)	Shioe, Kagawa	AB586663	Miyazaki et al. (2011)
(Nihonkai)	Natasyo Fukui	LC225600	Miyazaki et al. (2018)
(Nihonkai)	Oonyu, Kyoto	LC225605	Miyazaki et al. (2018)
<i>L. tokaiensis</i>			
NSMT-P132821 (holotype)	Kadoya, Aichi		Ito et al. (2019)
FAKU 211507–211511	Myoken, Aichi	LC790716	This study
FAKU 211512–211518	Kadoya, Aichi	LC790717, LC790718	This study
FAKU 211519–211526	Hosoe, Shizuoka	LC790719	This study
FAKU 211527	Ota, Shizuoka	LC790720	This study
	Shitara, Aichi	AB586682	Miyazaki et al. (2011)
	Hourai, Aichi	AB586683	Miyazaki et al. (2011)
	Gotengawa, Aichi	AB586684	Miyazaki et al. (2011)
<i>L. echigonia</i>			
(Hokuriku)	Ouchi, Akita	AB586665	Miyazaki et al. (2011)
(Hokuriku)	Nishime, Akita	AB586666	Miyazaki et al. (2011)
(Tohoku)	Matsushima, Miyagi	AB586668	Miyazaki et al. (2011)
(Tohoku)	Shionosaki, Fukushima	AB586669	Miyazaki et al. (2011)
(Yamagata)	Kawanishi, Yamagata	AB586686	Miyazaki et al. (2011)
(Yamagata)	Shinjo, Yamagata	AB586687	Miyazaki et al. (2011)
(Kinki)	Kurosaki, Ishikawa	AB586671	Miyazaki et al. (2011)
(Kinki)	Tsuruga, Fukui	AB586672	Miyazaki et al. (2011)
(Tokai)	Takegawa, Shizuoka	AB586674	Miyazaki et al. (2011)
(Yokai)	Shizuoka, Shizuoka	AB586685	Miyazaki et al. (2011)
(North-Kanto)	Kakuda, Miyagi	AB586676	Miyazaki et al. (2011)
(North-Kanto)	Kurobane, Tochigi	AB586677	Miyazaki et al. (2011)
(South-Kanto)	Kozakuragawa, Ibaraki	AB586679	Miyazaki et al. (2011)
(South-Kanto)	Maiokagawa, Kanagawa	AB586680	Miyazaki et al. (2011)
<i>L. costata</i>	Yanggile (Korea)	AB586655	Miyazaki et al. (2011)
<i>L. nikkonis</i>	Shibecha, Hokkaido	AB586654	Miyazaki et al. (2011)
<i>L. nishimurai</i> sp. nov.			
LBM 1210059189 (holotype)	Fukui, Fukui	LC790702	This study
LBM 1210059177–1210059180, 1210059188–1210059195 (paratypes)	Fukui, Fukui		This study
LBM 1210059181–1210059187 (paratypes)	Echizen, Fukui	LC790700, LC790701	This study
FAKU 211455–211457	Echizen, Fukui	LC790703–LC90705	This study
FAKU 211458–211460	Fukui, Fukui	LC790706–LC790708	This study

eight independent runs. After verifying the parameter estimates and convergence using Tracer v1.7.2 (Rambaut and Drummond 2009), the first million trees were discarded. Based on the phylogenetic relationships within the genus estimated by Miyazaki et al. (2011) using the ribosomal S7 subunit region, *L. nikkonis* and *L. costata* were set as outgroups for the present analysis.

The haplotype relationships of ribosomal S7 subunit gene were inferred by statistical parsimony (Clement et al. 2000) using PopART v1.7 (Leigh and Bryant 2015). The mean genetic distances among the *Lefua* species were estimated using the Kimura two-parameter (K2P) in MEGA v11.0.10 (Tamura et al. 2021) using the same datasets as in the phylogenetic analyses.





**Figure 1.** Map of central Japan showing the distribution of three *Lefua* species. Orange, *L. torrentis*; red, *L. tokaiensis*; purple, *L. nishimurai* sp. nov. The distribution of *L. torrentis* and *L. tokaiensis* were in accordance with Yamada (2015) and Nakajima and Uchiyama (2017).

### Morphological examination and analysis

The external morphological characters and vertebrae of *Lefua* specimens were examined using the methodologies outlined by Hosoya (1983) and Hubbs and Lagler (2004), respectively. The morphologies of *L. torrentis*, *L. tokaiensis*, and the new species were compared based on their morphological similarities indicated previously (Miyazaki et al. 2011; Ito et al. 2019; Nakajima et al. 2021). Moreover, Nishimura (2020) and Nakajima et al. (2021) highlighted that the new species has smaller, more dorsally positioned eyes (i.e. a smaller ratio of interorbital width to body width) compared to *L. torrentis* and *L. tokaiensis*. Accordingly, the head width and the ratio of interorbital width to body width were newly measured and calculated, in addition to the following 17 previously examined morphometric characters: standard length, head length, body depth, body width, depth of caudal peduncle, length of caudal peduncle, predorsal length, preanal length, prepelvic length, height of dorsal fin, length of dorsal fin base, height of anal fin, length of anal fin, pectoral fin length, snout length, orbit diameter, and interorbital width. These lengths were measured using a caliper (Popular Caliper 150 mm, Shinwa Rules Co., Ltd., Japan) to the nearest 0.1 mm.

The number of dorsal fin rays, anal fin rays, caudal fin rays, and vertebrae were counted using a CMBW-80 X-ray apparatus box (Softex Co., Ltd., Japan). The first four vertebrae with the Weberian Apparatus and one vertebra fused to the pleurostyle in the hypural complex were also counted. The last two fin rays of the dorsal and anal fins were counted as single rays because they were supported by a shared pterygiophore.

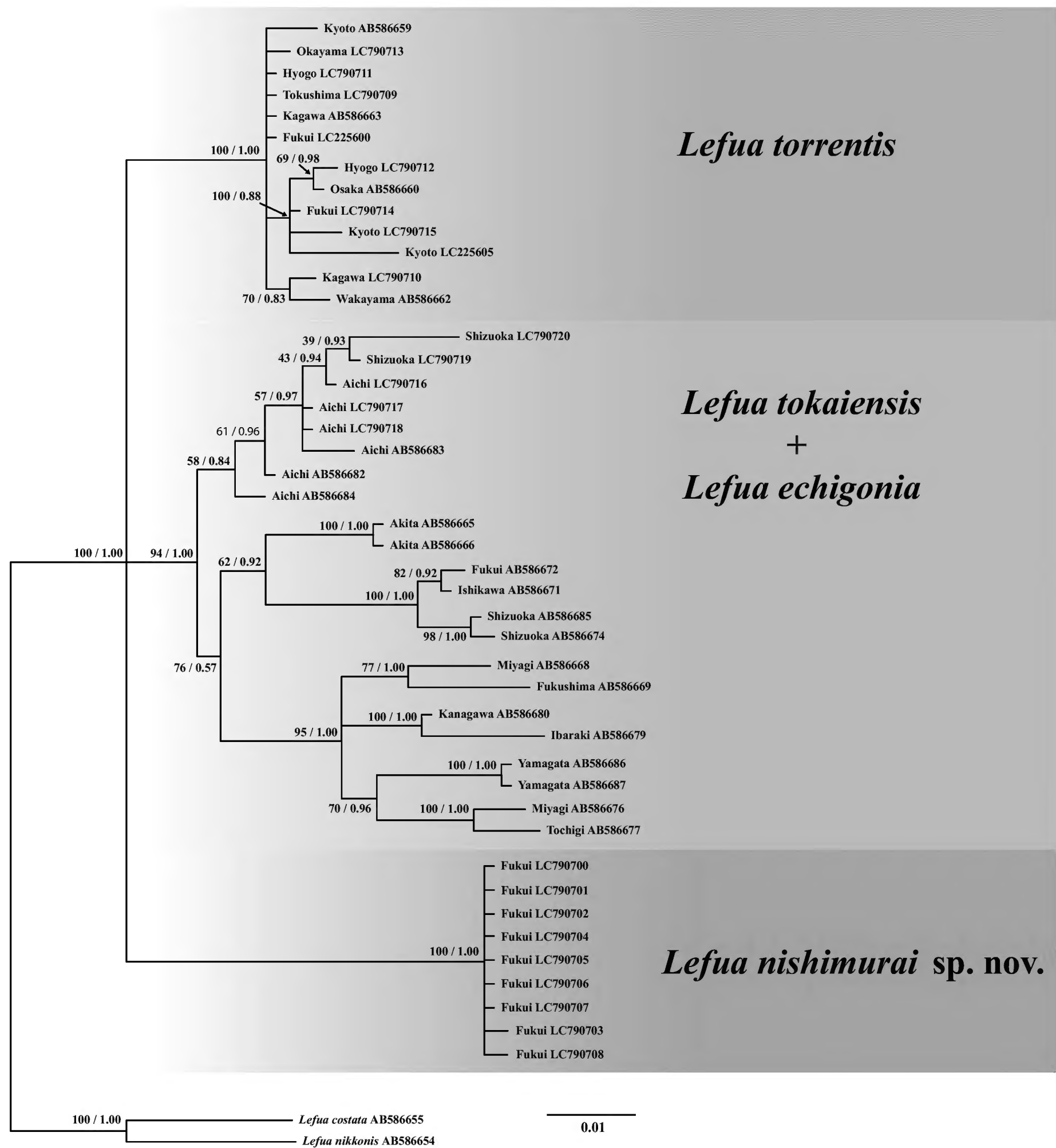
All characters of 19 type specimens of the new species were measured and counted. In non-types of the new species, *L. torrentis*, and *L. tokaiensis*, all characters were examined, except for the number of vertebrae and fin rays. In addition, 17 measurements and seven counts of holotypes of *L. torrentis* and *L. tokaiensis* were obtained from their original descriptions (Hosoya et al. 2018; Ito et al. 2019).

External morphological relationships among *Lefua* species were evaluated using principal component analysis (PCA). In addition, discriminant analysis was performed using the Random Forest (RF) algorithm (Breiman 2001). PCA and RF analyses were performed using standardized data for the 18 morphological characters of all newly collected specimens and holotypes of *L. torrentis* and *L. tokaiensis*. In the discriminant analysis, three variables were randomly sampled as candidates for each split, and 100,000 trees were generated, given that the out-of-bag error rate fully decreased with a large number of trees. PCA was performed using R v3.6.1 (R Development Core Team 2019). RF analyses were conducted using the package randomForest v4.6–14 (Andy and Matthew 2002) for R.

## Results

### Genetic analyses

The BI tree (Fig. 2) was identical to the ML tree (not shown). The reconstructed trees based on the 452 bp ribosomal S7 subunit gene supported the monophyly of *L. torrentis* [BS = 100, Bayesian posterior probabilities



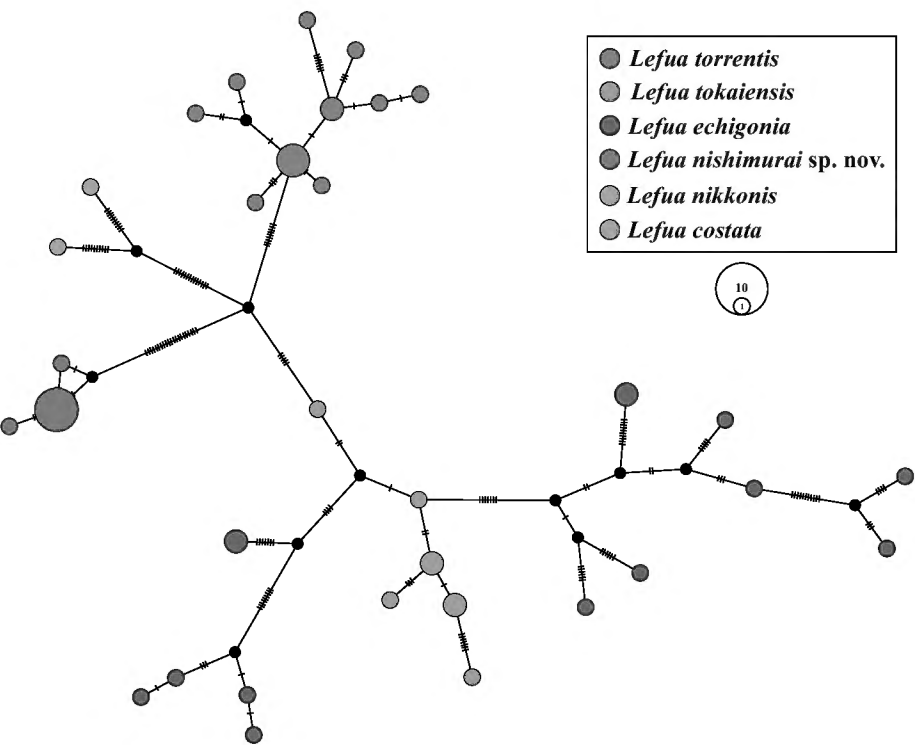
**Figure 2.** The Bayesian inference tree of six *Lefua* species based on 452 bp of the nucleic ribosomal S7 subunit gene. The numbers associated with the nodes represent the bootstrap (BS) values for ML/ and Bayesian posterior probabilities (BPP). BS higher than 50% and/or BPP higher than 90% are indicated.

(BPP) = 1.00) and *L. nishimurai* sp. nov. (BS = 100, BPP = 1.00), and the clade consisted of *L. tokaiensis* and *L. echigonia* (BS = 95, BPP = 1.00), respectively. Monophyly of the clade formed by these four species was also strongly supported (BS = 100, BPP = 1.00).

The statistical parsimony network calculated based on the 33 haplotypes separated all species, except for *L. tokaiensis* and *L. echigonia* by mutational steps (Fig. 3). In this network, the new species was separated from *L. torrentis* and *L. tokaiensis* by 31 and 27 steps,

respectively. Three haplotypes with a single-base substitution in each other were detected in nine specimens of *L. nishimurai* sp. nov.

*Lefua nishimurai* sp. nov. represented 0.1% intraspecific genetic differences, whereas *L. torrentis*, *L. tokaiensis*, and *L. echigonia* showed broader ranges of 0.8–4.9% (Table 2). The interspecific genetic differences among the four species were estimated to be 4.8–9.9% and the distances of *L. torrentis*, *L. tokaiensis*, and *L. echigonia* to *L. nishimurai* sp. nov. were 8.2%, 7.8%, and 9.9%, respectively.



**Figure 3.** Statistical parsimony network showing the relationships between the 33 haplotypes detected in a ribosomal S7 subunit gene of six *Lefua* species. Each connection indicates a single mutation, and hatch marks represent missing intermediate haplotypes.

Morphological analyses

PCA performed on *L. torrentis*, *L. tokaiensis*, and *L. nishimurai* sp. nov. using 18 external morphological characters summarized their morphological relationships.

The first two PCs explained 81.8% of the total variation, and PC2 scores primarily separated the three species with large overlaps between *L. torrentis* and *L. tokaiensis*, and slight overlaps between *L. nishimurai* sp. nov. and the other two species (Fig. 4). Among the morphological characters analyzed, standard, predorsal, and prepelvic lengths explained the variation of PC1 more strongly than the others (Suppl. material 1). For PC2, the contributions of the orbit diameter and snout length were greater than those of the other characters.

RF classification using the same dataset as PCA correctly classified 79.1% of the specimens into the three species. Bootstrap replicates identified 95.6% *L. torrentis*, 38.1% *L. tokaiensis*, and 84.0% *L. nishimurai* sp. nov. The mean decrease of the Gini coefficients was larger for interorbital width, snout length, orbit diameter, preanal length, body width, and head width (Table 3). The mean decrease in accuracy was greater for snout length, preanal length, interorbital width, body width, head width, and orbit diameter in *L. nishimurai* sp. nov. The specimens of the new species possessed a smaller snout length, ratio of interorbital width to body width, and interorbital width than those of *L. torrentis* and *L. tokaiensis*. The preanal length of the new species was greater than that of *L. torrentis* and *L. tokaiensis*. The body and head widths of the new species were larger than those of *L. torrentis* and smaller than those of *L. tokaiensis* (Figs 5, 6).

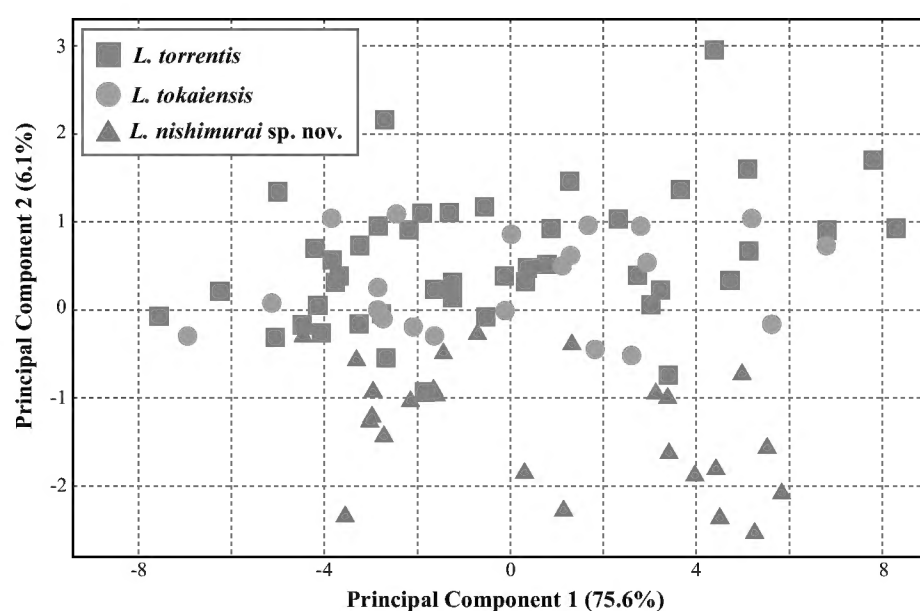
**Table 2.** Mean intraspecific (along the diagonal) and interspecific (below the diagonal) genetic distances (%) of six *Lefua* species calculated by a Kimura two-parameter model.

Species	<i>L. torrentis</i>	<i>L. tokaiensis</i>	<i>L. echigonia</i>	<i>L. nishimurai</i> sp. nov.	<i>L. nikkonis</i>	<i>L. costata</i>
<i>L. torrentis</i>	0.80					
<i>L. tokaiensis</i>	4.79	1.08				
<i>L. echigonia</i>	7.07	4.82	4.87			
<i>L. nishimurai</i> sp. nov.	8.17	7.81	9.90	0.10		
<i>L. costata</i>	8.09	7.35	10.06	11.37	–	
<i>L. nikkonis</i>	8.57	8.22	10.11	11.34	4.81	–

**Table 3.** Results of the Random Forest analyses for three *Lefua* species with specimen numbers, mean decrease in accuracy in each character, and the mean decreases in Gini coefficients.

Character	<i>L. torrentis</i>	<i>L. tokaiensis</i>	<i>L. nishimurai</i> sp. nov.	Mean decrease of Gini coefficient
Specimen number	45	21	19	
Body depth	0.007	0.020	0.001	2.100
Body width	0.042	0.053	0.004	4.067
Depth of caudal peduncle	0.014	0.011	0.014	2.420
Head length	0.010	0.010	-0.004	1.835
Head width	0.037	0.048	0.020	4.022
Height of anal fin	0.025	0.012	0.012	2.925
Height of dorsal fin	0.016	0.003	-0.002	2.907
Interorbital width	0.026	0.056	0.073	5.539
Length of anal fin base	0.005	0.003	0.003	1.480
Length of caudal peduncle	0.011	0.008	0.011	2.886
Length of dorsal fin base	0.011	0.009	0.001	2.274
Orbit diameter	0.030	0.045	0.013	4.874
Pectral fin length	0.005	0.002	0.004	1.636
Preanal length	0.031	0.057	0.021	4.292
Predosal length	0.017	0.034	0.009	2.915
Prepelvic length	0.016	0.026	0.003	2.549
Snout length	0.033	0.063	0.017	5.026
Standard length	0.014	0.017	0.012	2.659





**Figure 4.** Results of the principal component analysis based on the 18 external morphological characters conducted for three *Lefua* species.

## Systematics

### *Lefua nishimurai* Katayama, sp. nov.

<https://zoobank.org/7CD04DBC-DF52-4FD4-AEBC-FBDA719E800C>  
Table 4, Figs 5E, 6E, 7–9

New Standard Japanese name: Reihoku-nagare-hotoke-dojyô

*Lefua torrentis*: Natural Environment Division, Department of Safety and Environment, Fukui Prefecture 2016: 110 (part); Nakajima et al. 2021: 35, fig. 1; Katayama 2021: 54, fig. 5.

**Material examined. Holotype.** • LBM 1210059189; 51.2 mm SL; collected from Kuzuryu River system, Fukui, Fukui Prefecture, Japan; 36.0621°N, 136.1304°E; on 6 Sep. 2021 by Yuta Katayama.

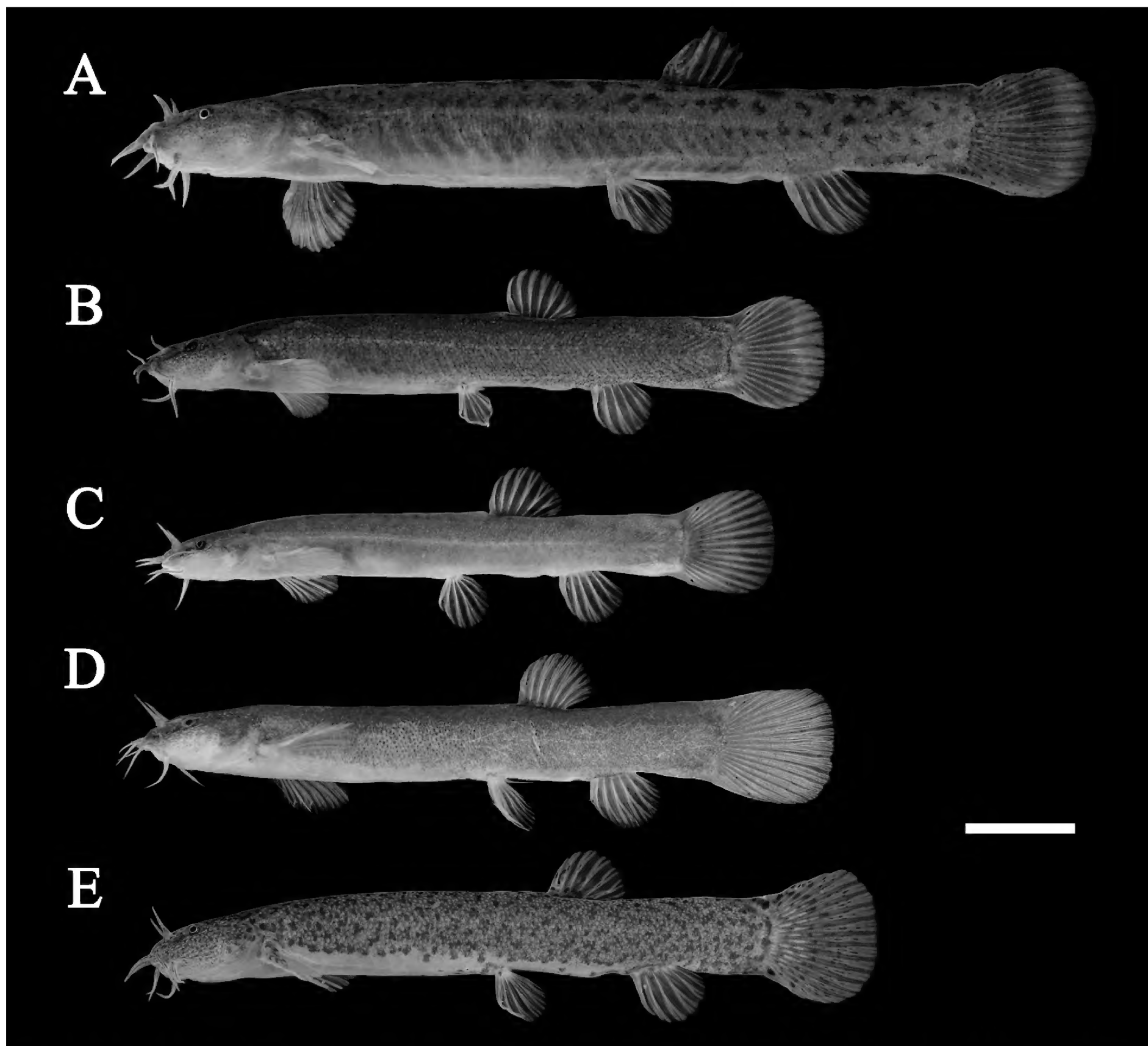
**Paratypes.** Eighteen specimens collected from Kuzuryu River system in Fukui Prefecture, Japan by Y. Katayama. • LBM 1210059177; 44.4 mm SL from Echizen on 17 Jul. 2021; • LBM 1210059178, 58.8 mm SL; • LBM 1210059179, 44.4 mm SL; • LBM 1210059180, 40.0 mm SL from Fukui on 18 Jul. 2021; • LBM 1210059181, 61.5 mm SL; • LBM 1210059182, 52.8 mm SL; • LBM 1210059183, 44.2 mm SL; • LBM 1210059184, 49.6 mm SL from Echizen on 17 Jul. 2021; • LBM 1210059185, 59.9 mm SL; • LBM 1210059186, 43.0 mm SL; • LBM 1210059187, 44.5 mm SL from Echizen on 6 Sep. 2021; • LBM 1210059188, 55.0 mm SL; • LBM 1210059190, 57.0 mm SL; • LBM 1210059191, 44.5 mm SL; • LBM 1210059192, 63.2 mm SL; • LBM 1210059193, 48.7 mm SL; • LBM 1210059194, 62.9 mm SL from Fukui on 6 Sep. 2021; • LBM 1210059195, 57.4 mm SL from Echizen on 13 Nov. 2021.

**Additional specimens.** Six specimens collected from Kuzuryu River system in Fukui Prefecture, Japan by Y. Katayama. • FAKU 211455, 41.5 mm SL; • FAKU 211456, 61.3 mm SL; • FAKU 211457, 56.1 mm SL from Echizen on 1 Oct. 2023; • FAKU 211458, 51.9 mm SL; • FAKU 211459, 47.3 mm SL; • FAKU 211460, 44.5 mm SL from Fukui on 1 Oct. 2023.

**Diagnosis.** *Lefua nishimurai* sp. nov. can distinguished from all other species of *Lefua* by combing following fea-

tures: absence of rhomboid or triangular dark blotches on middle of caudal fin base; absence of black longitudinal stripe on both body sides in mature males; absence of dusky cross bars on dorsal area of body; absence of dusky bar beside dorsal fin base; eyes located dorsally on head; narrow conspicuous longitudinal mark between base of outer rostral barbel and eye; small dorsal fin; small orbit diameter (6.3–11.2% of head length); small value of interorbital width relative to body width (28.1–39.4%); dark spots dorsally and ventrally on caudal fin base; small dark brown spots from snout to caudal peduncle; small dark spots on dorsal and caudal fins (approximately same size as eyes).

**Description.** Measurements and counts listed in Table 4. Body elongated and cylindrical, slightly longitudinally flattened, and caudal peduncle laterally flattened. Dorsal and ventral sides of caudal peduncle keeled, and depth of caudal peduncle, including keel, about same as body depth. Head small (less than one-fourth of SL), slightly dorsoventrally compressed. Snout relatively long, comprising 38.7% in holotype (32.3–46.4% in paratypes and additional specimens) of head length, with tip rounded in dorsal view. Eyes positioned on dorsolateral surface of head (Figs 6–8), very small. Interorbital width comprises 51.5% (42.9–59.0%) of body width, and orbit diameter comprises 6.6% (6.3–11.2%) of head length (Figs 6–8). Mouth subterminal; small and inferior, slightly arched. Upper and lower lips fleshly and smooth (Figs 7, 8). No median incision in upper lip. One median notch in lower lip. Three pairs of barbels, two pairs of rostral barbels, and one pair of maxillary barbels. Inner rostral barbel relatively short, reaching to or slightly past vertical through anterior margin of eye when extended horizontally, outer rostral barbel longest, slightly past vertical through posterior margin of eye when extended horizontally, maxillary barbel past vertical through posterior margin of eye when extended horizontally. Nostrils separated from each other, anterior nostril formed as 1 long nasal barbel, posterior nostril larger than anterior nostril, anterior nostril at anterior side of 1 nasal barbel, close to base. Nasal barbel extended horizontally past posterior border of eye. Dorsal and ventral keels on caudal peduncle; dorsal keel starting slightly posterior to extremity of dorsal fin base, ventral keel starting posterior to extremity of anal fin base. Length of caudal peduncle 1.0 (1.0–1.3) times depth of caudal peduncle (depth including keel). Scales embedded on body. Lateral line absent. Dorsal fin rounded. Origins of dorsal fin nearer to caudal fin base than to tip of snout. Pectoral fin horizontal. Pelvic fin anterior to dorsal fin. Anal fin rounded. Anus positioned slightly anterior to anal fin. Caudal fin rounded. Dorsal fin with 4 (3–4) simple and 5 (5–6) branched rays. Second branched ray longest. Anal fin with 4 (3–4) simple and 5 (5–6) branched rays. Pectoral fin with 1 simple and 11 (10–11) branched rays. Second branched ray longest. Pelvic fin with 1 simple and 5 branched rays. No pelvic axillary lobe. Caudal fin with 2 (1–2) simple, 6 (5–7) branched, 7 (6–7) branched and 1 (1–2) simple principal



**Figure 5.** Comparison of body morphology and coloration of three *Lefua* species. **A.** Kii-Shikoku Population of *L. torrentis* (FAKU 211486); **B.** Sanyo Population of *L. torrentis* (FAKU 211492); **C.** Nihonkai Population of *L. torrentis* (FAKU 211504); **D.** *L. tokaiensis* (FAKU 211510); **E.** *L. nishimurai* sp. nov. (LBM 1210059189). Scale bar: 10 mm.

rays. Total vertebrae 41 (39–43); abdominal vertebrae 23 (21–23), caudal vertebrae 18 (16–21).

**Coloration.** Live specimens, body and head light brown with strong yellowish tinge except for whitish gray ventral surface (Fig. 7). One narrow conspicuous black stripe between base of outer rostral barbel and eye (Fig. 8). Entire body from snout to caudal peduncle scattered dark brown spots. Largest of spots slightly larger than orbit diameter. Both lips white, with one pair of small brown spots inside lower lip tissue (Fig. 8). All fins pale to translucent. Pectoral fin yellowish brown, with dark spots at the base and anterior half. Dorsal fin yellowish brown with dark spots from base to middle. Anal fin with a few dark spots on basal half (sometimes absent). No spots on pelvic fin. Caudal fin yellowish brown with dark spots from base to a little closer to tip of middle, but translucent and without spots on margins. Dorsal and ventral surfaces of caudal

fin base with dark brown spots (rarely absent) (Figs 8, 9). No obscure dark band in skin of caudal fin base (rarely present) (Figs 5, 7, 9).

**Etymology.** The specific name is dedicated to Toshiaki Nishimura, who first morphologically distinguished this new species.

**Distribution.** This new species was recorded in the Kuzuryu River drainage system of the Reihoku Region in Fukui Prefecture, central Japan (Katayama 2021; Fig. 1).

**Habitat and biology.** *Lefua nishimurai* sp. nov. inhabits small mountain streams surrounded by natural or planted forests, and prefers sandy or gravelly bottoms (Fig. 10). The new species usually hides under rocks and stones at the river edge or under sediments, such as fallen leaves. It has been estimated that spawning of this species occurs from late April to mid-June (Katayama 2021).

**Remarks.** The new species is similar to *L. torrentis* and *L. tokaiensis* with a narrow conspicuous longitudi-

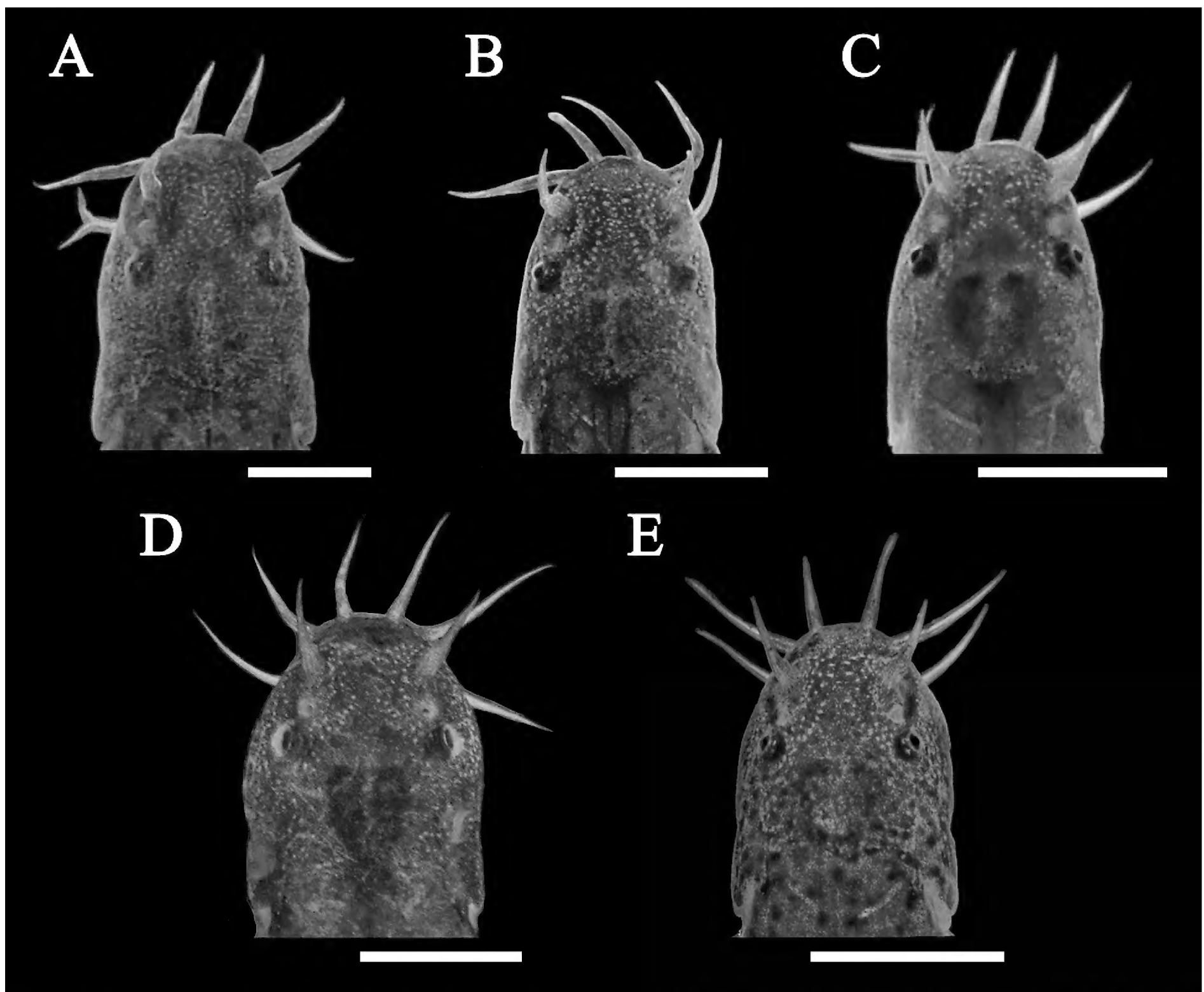


**Table 4.** Meristics and morphometrics of *Lefua torrentis*, *L. tokaiensis*, and *L. nishimurai* sp. nov. Measurements indicate minimum–maximum values (mean ± SD). Data of the holotypes of *L. torrentis* and *L. tokaiensis* were obtained from Hosoya et al. (2018) and Ito et al. (2019), respectively.

Character	<i>L. torrentis</i>		<i>L. tokaiensis</i>		<i>L. nishimurai</i> sp. nov.		
	Holotype	Non-types (n = 46)	Holotype	Non-types (n = 21)	Holotype	Paratypes (n = 18)	Non-types (n = 6)
Dorsal fin	iv, 6		iv, 6		iv, 5	iii–iv, 5–6	
Anal fin	iv, 5		iv, 5		iv, 5	iii–iv, 5–6	
Pectral fin	i, 9		i, 7		i, 11	i, 10–11	
Pelvic fin	i, 5		i, 5		i, 5	i, 5	
Caudal fin	ii, 6, 8, i		i, 7, 8, ii		ii, 6, 7, i	i–ii, 5–7, 6–7, i–ii	
Abdominal vertebrae	19		19		23	21–23	
Caudal vertebrae	18		18		18	16–21	
Total vertebrae	37		37		41	39–43	
Standard length (mm)	44.9		44.2		51.2	40.0–63.2	
In % of standard length							
Head length	20.9	18.4–23.4 (21.0 ± 1.2)	21.3	19.1–26.4 (22.0 ± 1.9)	20.7	19.1–23.6 (20.6 ± 0.9)	19.6–22.0 (20.8 ± 1.0)
Body depth	14.4	8.8–15.2 (12.1 ± 1.2)	14	10.8–17.1 (13.6 ± 1.5)	12.7	10.7–14.2 (12.4 ± 1.0)	11.8–14.3 (13.1 ± 0.9)
Body width	13.9	11.2–14.7 (12.7 ± 0.8)	11.7	12.3–17.2 (14.2 ± 1.4)	13.3	12.5–14.6 (13.6 ± 0.6)	12.9–14.2 (13.6 ± 0.4)
Head width		11.2–15.9 (13.2 ± 1.0)		13.0–17.6 (15.2 ± 1.4)	13.1	12.5–15.2 (13.5 ± 0.6)	12.9–14.4 (13.7 ± 0.5)
Depth of caudal peduncle	12.4	10.8–13.3 (12.1 ± 0.7)	13.2	11.0–17.1 (13.7 ± 1.4)	12.5	10.7–13.5 (12.0 ± 0.7)	11.6–13.0 (12.2 ± 0.5)
Length of caudal peduncle	14.8	11.7–16.8 (14.4 ± 1.1)	16.1	11.3–116.2 (13.7 ± 1.1)	12.7	11.7–15.2 (13.5 ± 0.9)	11.9–14.9 (13.3 ± 0.9)
Predorsal length	67.2	61.5–69.1 (64.8 ± 1.6)	62.9	62.8–76.9 (66.3 ± 3.1)	67.2	63.2–69.3 (66.9 ± 1.3)	65.3–68.1 (67.1 ± 0.9)
Preanal length	76	71.2–80.8 (75.0 ± 1.6)	74.8	72.3–87.0 (75.9 ± 3.1)	77.9	76.6–81.4 (79.3 ± 1.3)	74.3–82.2 (77.1 ± 2.4)
Prepelvic length	56.1	53.3–65.2 (57.0 ± 1.9)	53.6	56.5–74.0 (60.1 ± 4.2)	56.8	53.6–60.9 (57.6 ± 1.7)	56.8–60.5 (58.5 ± 1.1)
Height of dorsal fin	10.9	6.6–11.7 (9.2 ± 1.1)	11.3	6.1–11.8 (8.8 ± 1.3)	8.6	5.6–10.0 (7.9 ± 1.1)	7.0–9.6 (8.5 ± 1.0)
Length of dorsal fin base	8.1	6.7–10.1 (8.6 ± 0.7)	8.6	7.1–9.9 (8.5 ± 0.8)	8.2	7.5–9.7 (8.6 ± 0.6)	7.2–9.0 (8.4 ± 0.6)
Height of anal fin	12.5	5.5–10.7 (7.7 ± 1.1)	10.3	7.0–11.3 (8.7 ± 1.0)	9.4	6.5–9.3 (7.9 ± 0.9)	5.5–9.0 (7.7 ± 1.1)
Length of analfin base	8.1	6.8–9.9 (8.9 ± 0.6)	6.6	6.8–11.9 (8.2 ± 1.0)	7.2	5.8–9.1 (7.8 ± 0.8)	7.0–8.3 (7.6 ± 0.4)
Pectral fin length	13.6	11.6–17.0 (14.1 ± 1.4)	12.4	13.0–18.6 (15.1 ± 1.4)	12.9	11.2–16.5 (13.1 ± 1.4)	12.5–15.1 (13.5 ± 0.9)
In % of head length							
Snout length	34	41.6–53.8 (48.9 ± 3.3)	39.7	44.5–55.5 (49.4 ± 3.4)	38.7	32.3–45.7 (40.1 ± 3.4)	42.2–46.4 (44.6 ± 1.5)
Orbit diameter	13	6.9–15.9 (10.5 ± 1.7)	13.6	7.3–13.3 (9.5 ± 1.4)	6.6	6.3–11.2 (8.1 ± 1.5)	7.5–9.4 (8.5 ± 0.6)
Interorbital width	39	32.2–47.3 (39.6 ± 3.8)	36.3	39.3–48.9 (43.8 ± 2.7)	33.0	28.1–39.4 (34.5 ± 3.2)	33.3–39.1 (36.8 ± 1.8)
Ratio of interorbital width to body width	58.6	55.9–82.1 (65.6 ± 6.0)	66.1	56.3–76.4 (68.0 ± 6.2)	51.5	42.9–58.2 (52.2 ± 4.3)	51.6–59.0 (56.1 ± 3.0)

nal mark between the base of the outer rostral barbel and eye. However, *L. nishimurai* sp. nov. can be distinguished from *L. torrentis* based on the following characteristics: eyes located dorsally on head (more lateral in *L. torrentis*), small orbit diameter (larger in *L. torrentis*), dark spots on the caudal fin base (rarely absent in the new species; usually absent in *L. torrentis*), small dark spots on dorsal and caudal fins, and distinct dark brown spots scattered over the body (rarely absent in the new species; entirely

absent in *L. torrentis*). The new species and *L. tokaiensis* can also be discriminated by eyes located dorsally on the head (more lateral in *L. tokaiensis*), small orbit diameter (larger in *L. tokaiensis*), dark spots dorsally and ventrally on the caudal fin base (rarely absent in the new species; entirely absent in *L. tokaiensis*), distinct dark brown spots scattered over the body (rarely absent in the new species; entirely absent in *L. tokaiensis*), and fan-shaped caudal fin of *L. nishimurai* (squared-off apex in *L. tokaiensis*).



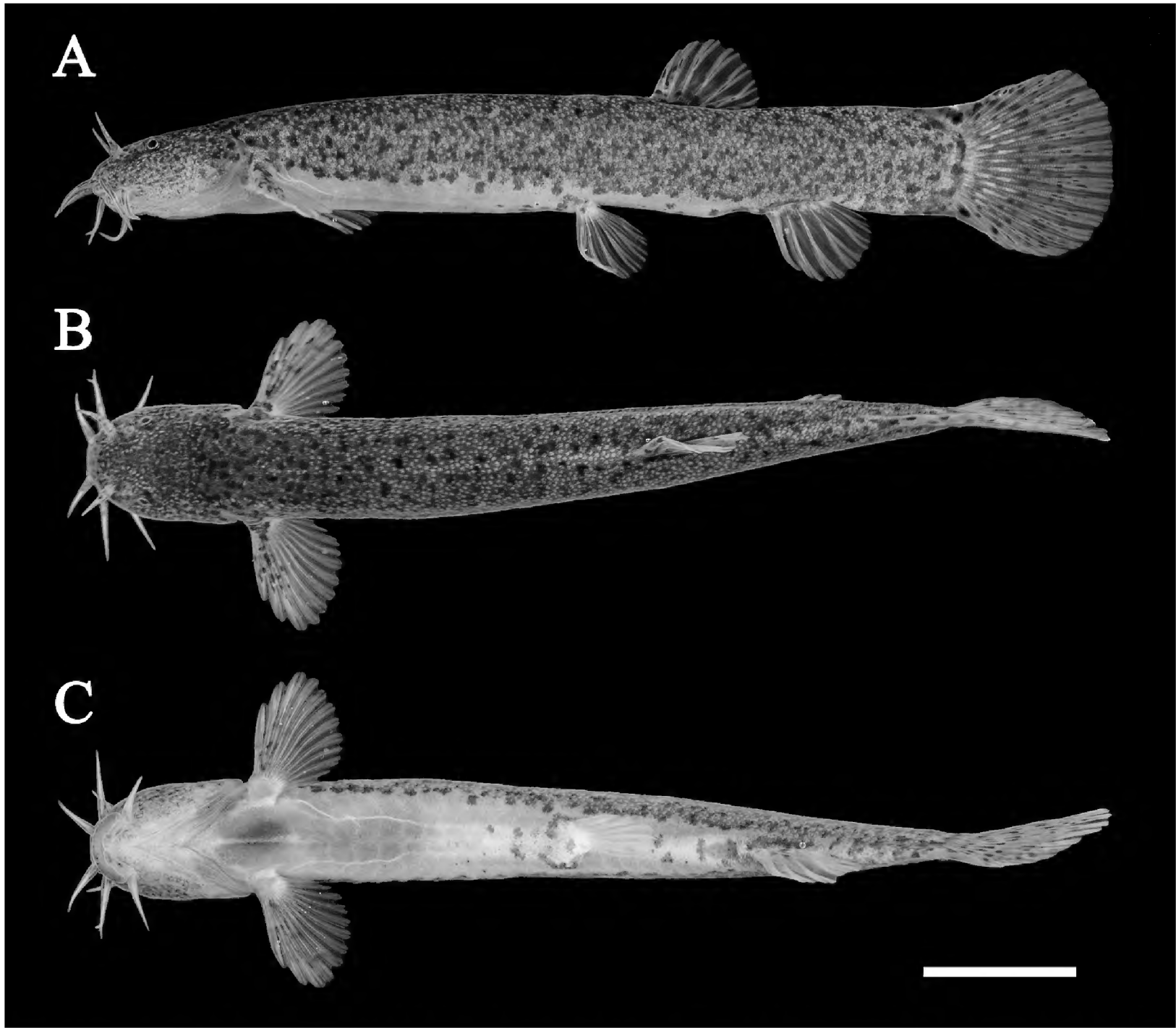
**Figure 6.** Comparison of dorsal view of the head of three *Lefua* species. **A.** Kii-Shikoku Population of *L. torrentis* (FAKU 211486); **B.** Sanyo Population of *L. torrentis* (FAKU 211492); **C.** Nihonkai Population of *L. torrentis* (FAKU 211504); **D** *L. tokaiensis* (FAKU 211510); **E.** *L. nishimurai* sp. nov. (LBM 1210059179). Scale bars: 5 mm.

Both the new species and *L. torrentis* inhabiting Wakayama Prefecture, and Shikoku and Awaji Islands possess dark brown spots of varying sizes on the body, dorsal side, and caudal peduncle (Hosoya et al. 2018). However, *L. nishimurai* sp. nov. represents smaller spots than the other species on the entire body and dorsal surfaces as well as on the dorsal and caudal fins (approximately the same size as the eyes) (Fig. 5).

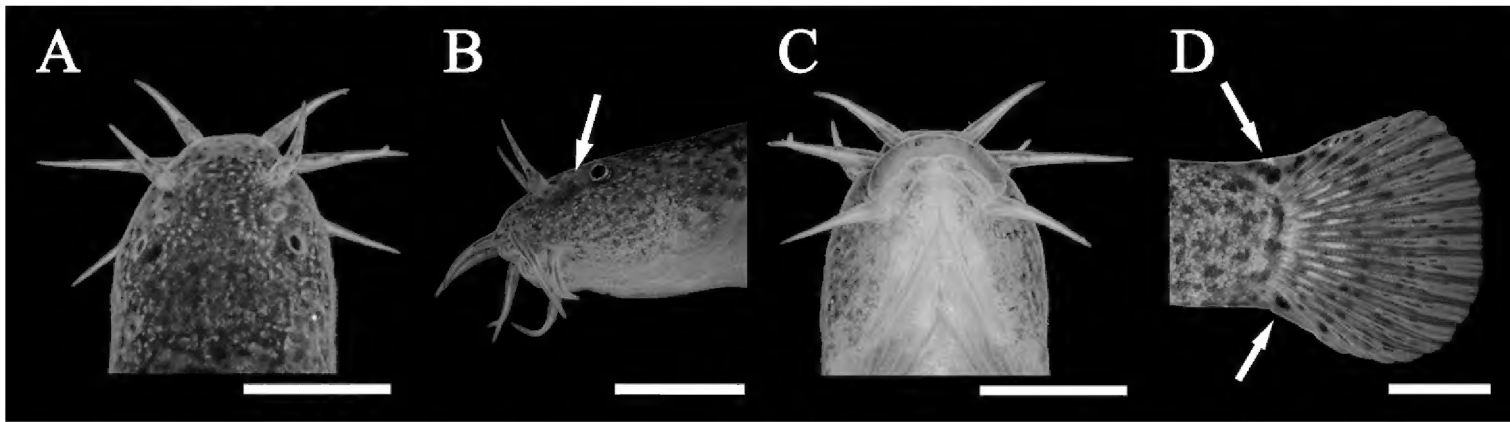
**Comparative material examined.** *Lefua torrentis*, 46 specimens (33.2–66.9 mm SL). **Holotype:** • KUN-P 45408; 44.9 mm SL collected from Yura River system, Kasuga, Tanba, Hyogo; • FAKU 211461–211464, 4 specimens, 33.2–49.4 mm SL from Arida River system, Aridagawa, Wakayama; • FAKU 211465–211468, 4 specimens, 43.4–55.5 mm SL, from Hidaka River system, Hidaka, Wakayama; • FAKU 211469–211477, 9 specimens, 38.4–49.9 mm SL from Orino River system, Kitanada, Tokushima; • FAKU 211478–211480, 3 specimens, 42.9–55.5 mm SL from Yoshino River system, Gomyo, Kagawa; • FAKU 211481–211484, 4 specimens, 40.4–63.6 mm SL from Sumoto River system,

Ayuya, Hyogo; • FAKU 211485–211490, 6 specimens, 44.2–69.9 mm SL from Dainichi River system, Jindai-urakabe, Hyogo; • FAKU 211491, 1 specimen, 49.6 mm SL from Muko River system, Dojyo, Hyogo; • FAKU 211492, 1 specimen, 40.0 mm SL from Yoshii River system, Nagi, Okayama; • FAKU 211493–211499, 7 specimens, 39.0–60.0 mm SL from Maruyama River system, Santo, Hyogo; • FAKU 211500, 1 specimen, 44.9 mm SL from Saburi River system, Yamada, Fukui; • FAKU 211501–211506, 6 specimens, 35.6–57.4 mm SL from Onyu River system, Taneji, Kyoto.

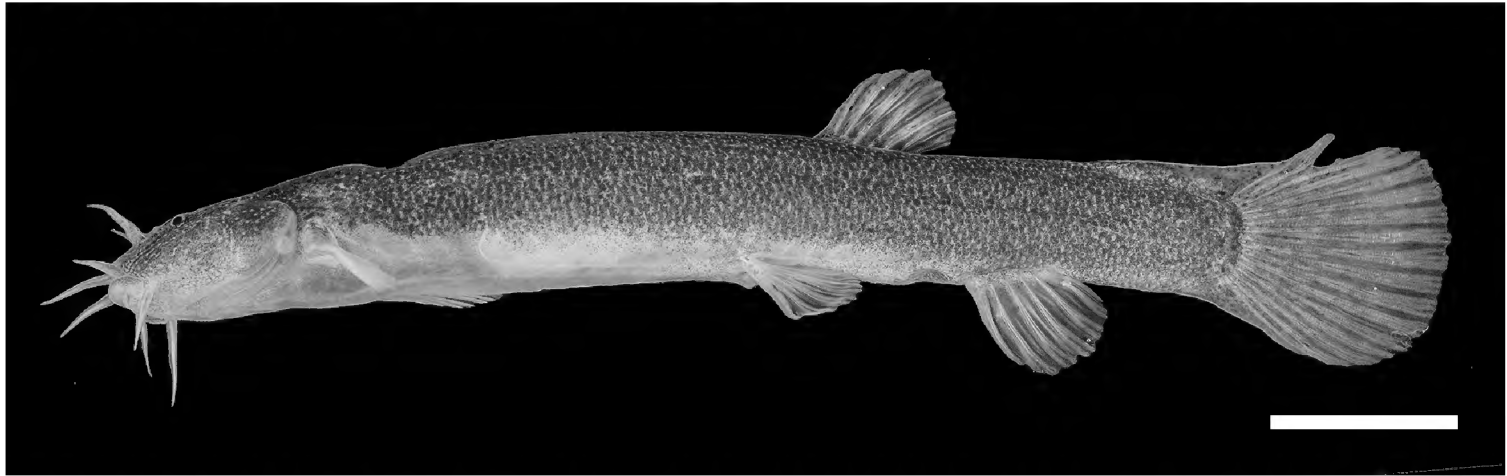
*L. tokaiensis*, 22 specimens (32.8–60.9 mm SL). **Holotype:** • NSMT-P 132821, 44.2 mm SL from Toyo River, Kadoya, Aichi; • FAKU 211507–211511, 5 specimens, 32.8–51.3 mm SL from Yahagi River system, Myoken, Aichi; • FAKU 211512–211518, 7 specimens, 33.8–54.4 mm SL from Toyo River system, Kadoya, Aichi; • FAKU 211519–211526, 8 specimens, 40.7–60.0 mm SL from Miyakoda River system, Hosoe, Shizuoka; • FAKU 211527, 1 specimen, 36.8 mm SL from Ota River system, Ota, Shizuoka.



**Figure 7.** Body coloration of the holotype of *Lefua nishimurai* sp. nov (LBM 1210059189). **A.** Lateral view; **B.** Dorsal view; **C.** Ventral view. Scale bar: 10 mm.



**Figure 8.** Detailed morphology of the holotype of *Lefua nishimurai* sp. nov (LBM 1210059189). **A.** Head; **B.** Lateral view of head; **C.** Mouth; **D.** Caudal fin base. An arrow in the lateral view of head (**B**) indicates narrow conspicuous black stripe between base of outer rostral barbel and eye. Arrows in the caudal fin base (**D**) indicate black spots on ventral and dorsal surfaces. Scale bars: 5 mm.



**Figure 9.** Body coloration of atypical specimen of *Lefua nishimurai* sp. nov. (LBM 1210059188), showing no distinct dark brown spots scattered over the body and obscure dark band in the skin of the caudal fin base. Scale bar: 10 mm.





**Figure 10.** Typical habitat of *Lefua nishimurai* sp. nov. in the Kuzuryu River drainage system in Fukui Prefecture, Japan.

## Discussion

The genetic and morphological distinctiveness of *L. nishimurai* sp. nov. in the Kuzuryu River system was clarified through integrative analyses. The phylogenetic relationships among the *Lefua* species corresponded to those estimated in previous studies (Miyazaki et al. 2011, 2018). The reconstructed tree strongly supported the monophyly of *L. nishimurai* sp. nov. In addition, the statistical parsimony network showed that the new species possesses unique haplotypes of the ribosomal S7 subunit gene in the Japanese *Lefua* species.

The long-branched clade consisting of the new species and the large interspecific genetic distances between the new species and other congeners were estimated in this study. Although only one nucleic locus was analyzed in this study, these results suggest that the new species was genetically isolated at an early stage of diversification of the common ancestors of *L. torrentis*, *L. tokaiensis*, *L. echigonia*, and *L. nishimurai* sp. nov. In addition, the observed low haplotypic diversity may indicate past population size declines in this new species. In Fukui Prefecture, populations of freshwater fishes, such as *Pungtungia herzi* Herzenstein and *Tanakia limbata* (Temminck & Schlegel), have been geographically isolated between the northern Reihoku and the southern Reinan Regions (Fig. 1; Kato 1906; Igarashi and Kato 1966; Kato 1985, 1998; Matsumiya 2001). Therefore, the restricted distribution of the new species in the Reihoku Region might be due to the high altitudes surrounding the region, with patterns similar to those of other fish populations.

The incongruence between mitochondrial and nuclear phylogenies caused by historical mitochondrial introgression has been estimated in Japanese *Lefua* species (Miyazaki et al. 2018). The Nihonkai Population of *L. torrentis* in the Tango, Tajima, and Reinan Regions represents

mitochondrial haplotypes closely related to *L. echigonia* and *L. tokaiensis*, suggesting past hybridization among them or their ancestors (Miyazaki et al. 2018). A hybrid population of *Oryzias* species was also found in this area, and their hybridization has been attributed to the topographic features of the watershed boundary between the Paleo-Yura River and the Paleo-Kako River (Iguchi et al. 2018).

Given that the distribution of *L. echigonia* extends along the Sea of Japan (Nihonkai) (Miyazaki et al. 2018), the mitochondrial phylogeny of *Lefua* in Fukui Prefecture can be incorrectly estimated because of populations influenced by introgression. On the other hand, the genetic distinctiveness of *L. nishimurai* sp. nov. was clarified with mitochondrial and nuclear genes by Nakajima et al. (2021) and this study. Based on the genetic and morphological distinctiveness of the *Lefua* population in the Kuzuryu River system of the Reihoku Region, we consider *L. nishimurai* sp. nov. to be an independent species.

The characters of standard, predorsal, prepelvic, and head lengths contributed more strongly to explaining the morphological variation among specimens in the PCA. In contrast, interorbital width, snout length, and orbit diameter were more important in explaining interspecific variations in PCA and/or discriminant analysis. These results show that both previous and newly obtained characters are useful for discriminating the three *Lefua* species. The snout length, preanal length, interorbital width, body width, head width, and orbit diameter were particularly important for the identification of *L. nishimurai* sp. nov. in the RF classification. According to this analysis, the specimens of this new species can be discriminated by head morphology, which is characterized by more dorsally positioned small eyes and shorter snouts. The preanal length, and body and head widths of the new species are also larger than those of *L. torrentis* and smaller than those of *L. tokaiensis*.

According to prefectural surveys, a decrease in the number of freshwater fishes and salamanders, such as *Cottus pollux* Günther, *Hynobius abei* Sato, and *H. kimurae* Dunn, whose habitats potentially overlap with those of *L. nishimurai* sp. nov., has been progressing due to recent habitat degradation in the Reihoku Region (Natural Environment Division, Department of Safety and Environment, Fukui Prefecture 2016). Given the restricted distribution and vulnerable habitats of this new species, it is important to accumulate further knowledge on its distribution, population size, and natural history, as well as investigate its conservation status.

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## Supplementary material 1

### Loadings of the 18 external morphological characters, proportion of variance, and cumulative proportion for the 18 principal components (PC) calculated by the principal component analysis

Authors: Yuta Katayama, Naoto Sawada

Data type: docx

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